IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Kristian Berg et al.	Examiner: Gerald Ewoldt	
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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Dr. Anders Høgset, a Norwegian citizen, hereby declare as follows:

1. I am the Chief Scientific Officer of PCI Biotech AS, the assignee of the application in issue. I am also an inventor on the application in issue.

2. Additional experiments have been carried out under my direction that relate to the subject matter of the above-identified application. The results of these experiments are described in the attached Annex, and briefly summarized below.

3. *In vitro* and *in vivo* experiments were conducted to determine the effect of antigen internalization by photochemical internalization (PCI) methods on immune responses using Amphinex (which contains the photosensitizer $TPCS_{2a}$ in solution) as the photosensitizer and ovalbumin or its peptides as the antigen. The *in vitro* data show that after illumination, macrophages treated with Amphinex and ovalbumin peptides activate CD8+ T cells *in vitro*. However, this effect was not observed for MHC class II antigen presentation and CD4+ T cell activation. Thus the effect occurs via the MHC class I antigen presentation mechanism. These results were confirmed *in vivo* where it was observed that Amphinex and ovalbumin peptide administration to mice and activation by illumination resulted in a CD8+ T cell response in both spleen cells and blood cells. The activated CD8+ T cells were specific for the antigen used in immunisation as shown by detection of T cells specific to the peptide using relevant peptide-loaded MHC class I multimers. The results therefore show that photochemical internalization methods stimulate the generation of an immune response *in vivo*.

4. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such wilful false statements may jeopardize the validity of the application and any patent issuing thereon.

Anders Høgset 20TH SEPTEMBER 2011

Dr Anders Høgset Date

Application. No.: 09/524,454

ANNEX TO DECLARATION : EXPERIMENTAL DETAILS

SUMMARY

In vitro and *in vivo* experiments were conducted to determine the effect of antigen internalization using PCI methods on immune responses using Amphinex (which contains the photosensitizer $TPCS_{2a}$ in solution) as the photosensitizer and ovalbumin or its peptides as the antigen. The *in vitro* data show that macrophages treated with Amphinex and ovalbumin peptide (and illuminated to activate Amphinex) activate CD8+ T cells *in vitro*. However, this effect was not observed for MHC class II antigen presentation and CD4+ T cell activation and thus the effect clearly occurs via the MHC class I antigen presentation mechanism. These results were confirmed *in vivo* where it was observed that Amphinex and ovalbumin peptide administration to mice and activation by illumination resulted in a CD8+ T cell response in both spleen cells and blood cells and that the activated CD8+ T cells were specific for the antigen used in immunisation.

METHODS

Antigen-presenting cells
Bone-marrow derived macrophages (BMDMs), bone-marrow derived dendritic cells (BMDCs) from mice with C57/BL6 background as well as C57/BL6 derived macrophage cell lines were used as antigen-presenting cells (APCs). APCs were cultivated in RPMI (Sigma) medium supplemented with 10% FCS. BMDMs were generated by cultivating mouse bone-marrow cells for at least 5 days in medium supplemented with 20% L-292 cell line supernatant. Immature BMDCs were generated by cultivating murine bone-marrow cells for 6-8 days in 6-well plates ($5x10^5$/well) in the presence of 30 ng/ml GM-CSF (day 1, 3, 5). Identity of BMDCs was controlled by analyzing CD11c-expression with flow-cytometry.

Viability of cells
Survival of Amphinex + light treated APCs was controlled in most experiments. Viability was either controlled in a standard MTT cell proliferation assay or with flow-cytometry by identifying viable cells due to their forward (FSC) and sideward (SSC) scatter properties.

In vitro T cell activation assay
BMDMs, immature BMDCs and two murine macrophage cell lines (C57BL/6 background) were used as antigen-presenting cells (APCs) in an antigen-specific T cell setting with ovalbumin (peptide)-specific T cell hybridomas.

APCs were incubated with or without Amphinex in the indicated concentration overnight in a 96-well plate. Amphinex was then washed away and APCs incubated with different concentrations of antigen for 4h. Antigens used for stimulation were ovalbumin (OVA) peptides (OVA 257-264 and OVA 323-339, both from Anaspec) or a low-endotoxin preparation of recombinant OVA protein (Hyglos).

Cells were washed and exposed to different doses of blue light before ovalbumin-specific T cells were added and incubated overnight. The CD8+ T cell clone RF33.70

(MHC I-restricted recognition of OVA 257-264) and the CD4+ T cell clone MF2.2D9 (MHC II-restricted recognition of OVA 323-339) were used.

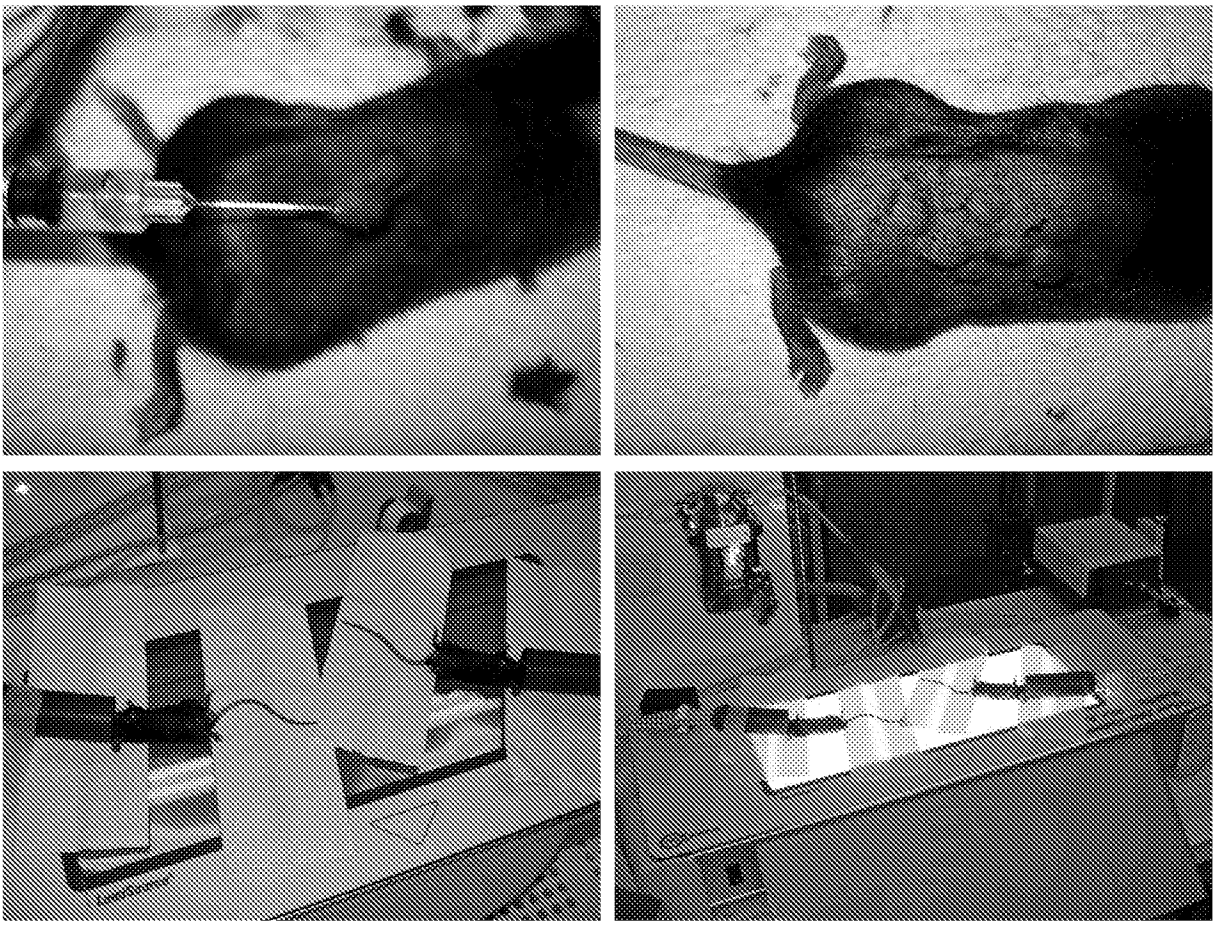
After overnight co-culture of T cells and APCs, supernatants from the cell culture were harvested. The supernatants were analyzed for interleukin (IL)-2 production from activated T cells by analyzing the proliferation of the IL-2-dependent T cell line HT-2 in a standard 3H-thymidine incorporation assay in the presence of supernatant from the APC-T cell co-culture.

Animals and immunization
C57BL/6 mice were purchased from Bomholdt (Denmark), some of the mice were bred at the animal facility at the Norwegian University of Science and technology.. The experiments were covered by an ongoing approval from the local animal care committee.
Day -1: Immunization of mice with the indicated Amphinex-concentration and antigen (OVA 257-264-peptide, 100 µg) in a total volume of 100µl PBS. Mice were anaesthesized (use of nitrous oxide + isofluorane) and shaved on the right side of their back. An insulin-syringe was used for intra-dermal (i.d.) injection of antigen +/- Amphinex (Figure 1A). A total volume of 100 µl was injected per mouse, distributed between 4-5 injection sites (20-25 µl per injection site) resulting in the formation of "bubbles" in the mouse skin (Figure 1B). Mice were kept overnight in the dark.
Day 0: Ca. 20 h after immunization mice were again anaesthesized, the injection-site was positioned on the LumiSource™ illumination device (PCI Biotech AS) and the mice were exposed 3-12 min to blue light (Figure 1C and 1D). Mice were kept for an additional 2-3 days in the dark and inspected daily.
Day 6-10: mice were sacrificed and T cell functions were analyzed from blood and spleen, in one experiment mice received booster immunizations on day 8 and 14 and T cells were analyzed on day 22.

Analysis of antigen-specific T cells from in vivo experiments
Mice were killed at the indicated time-point post infection. Spleens were removed aseptically, heparin-blood samples were obtained by heart-puncture.
After preparing single-cell suspensions from spleens and lysis of red blood cells (RBC lysis buffer, eBioscience) from blood and spleen samples, cells were analyzed by multi-color flow-cytometry on a BD LSR II flow-cytometer.

Staining for antigen-specific T cells was performed using peptide-loaded MHC class I multimers (H-2Kb/SIINFEKL Pro 5 Pentamer PE, ProImmune, UK), binding exclusively the T cell receptor of CD8+ T cells specific for the OVA 257-264 peptide that was used for immunization. Cells were further phenotyped using fluorescent monoclonal antibodies for the surface epitopes CD3, CD8, CD19, CD44 and CD25 (all from eBioscience). In *in vivo* experiment Nr. 4, additional CD8+ T cell functions were analyzed such as T cell proliferation in response to OVA-antigen (flow-cytometry).

Figure 1: Illustration of the intra-dermal immunization with Amphinex and blue-light treatment of the mice.



A) Intra-dermal injection; B) "bubbles" in the mouse skin after i.d. injection; C) mice were anaesthetized and positioned on the lamp ca. 20h post i.d. immunization; D) exposure of the injection site to blue-light.

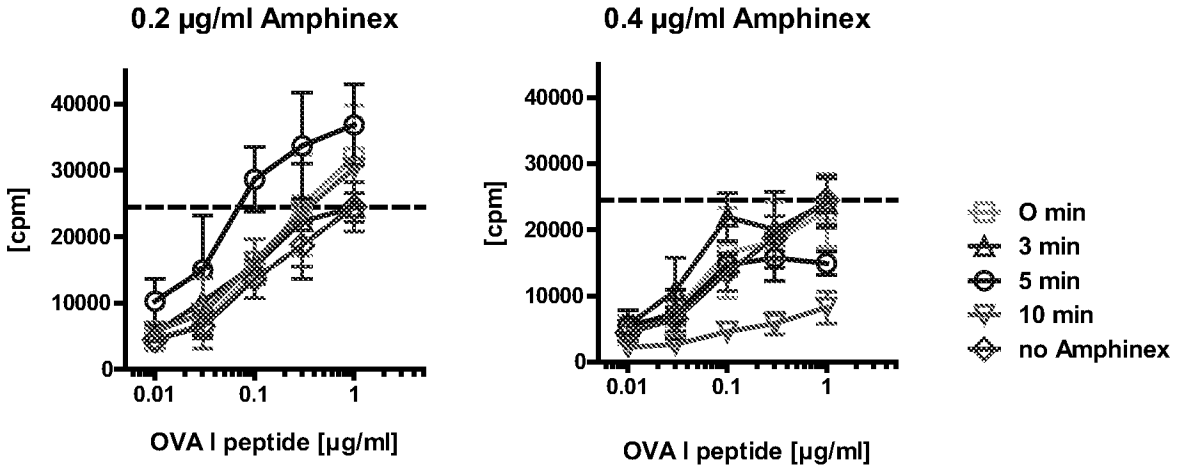
RESULTS

A. *IN VITRO EXPERIMENTS*

1. Amphinex enhances activation of antigen-specific CD8+ T cells

We analyzed the effect of Amphinex on antigen-presentation and T cell activation in an *in vitro* model using ovalbumin-derived antigens and specific CD4+ and CD8+ T cell clones. For testing of the Amphinex-effect on MHC class I-restricted antigen-presentation and CD8+ T cell activation, APCs were incubated with Amphinex and OVA 257-264 peptide as described in the methods in an antigen-specific T cell setting with an ovalbumin-specific (OVA 257-264) CD8+ T cell clone. Two murine macrophage cell lines (C57Bl/6 background), primary BMDMs as well as BMDCs were used as antigen-presenting cells (APCs) in these assays.

Figure 2: Effect of Amphinex on antigen-specific CD8+ T cell activation from macrophages.



Amphinex-enhanced CD8+ T cell activation using primary mouse macrophages (BMDMs) and OVA peptide antigen as described in methods. In brief, APCs were incubated with
A) 0.2 µg/ml Amphinex
B) 0.4 µg/ml Amphinex overnight.
Amphinex was then washed away and APCs incubated with different concentrations of OVA 257-264 peptide for 4h. Cells were washed and treated with different doses of light. The T cells were added and production of IL-2 from activated T cells was analyzed from the supernatant. The dotted line in the diagram indicates the T cell response achieved with 1 µg/ml antigen alone (no Amphinex).

We found a positive effect of Amphinex on MHC class I antigen-presentation and CD8+ T cell activation in an antigen-specific T cell setting with an ovalbumin-specific (OVA 257-264) CD8+ T cell clone. No CD8+ T cell activation was observed with the intact protein in any of the experiments (with or without Amphinex), data not shown.

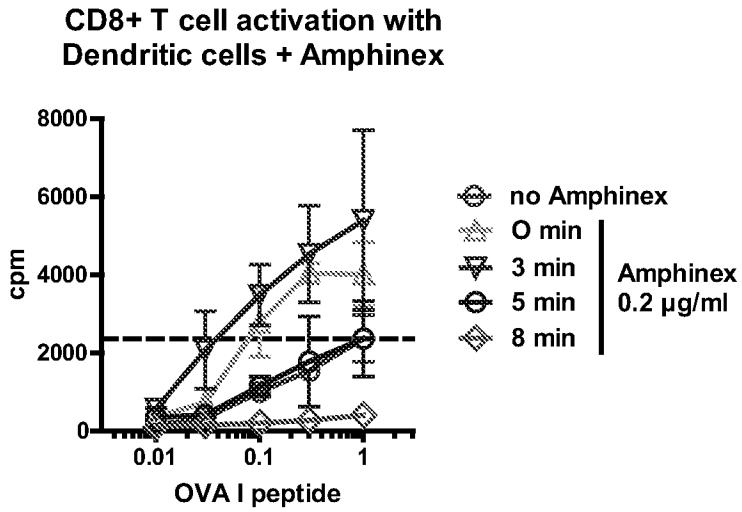
We found the best effect with BMDMs with 0.2 µg/ml Amph + 5 min.

With the higher Amphinex-doses (0.4 µg/ml) there was a minimal effect with reduced light-dose (3 min), but no effect at 5 min and almost no T cell activation with 10 min (probably due to cell death of APCs). The Amphinex-effect seems to be concentration and light-dose dependent.

We found a 10-100-fold enhanced CD8+ T cell activation using Amphinex + light in *in vitro* T cell stimulation experiments.

This effect was observed in several (more than 5) independent experiments and with different types of APCs. APCs used in these experiments were primary BMDMs (Figure 2), 2 different mouse macrophage cell lines (C57/BL5 background, not shown) as well as dendritic cells (BMDCs, Figure 3).

Figure 3: Effect of Amphinex on antigen-specific CD8+ T cell activation from dendritic cells.



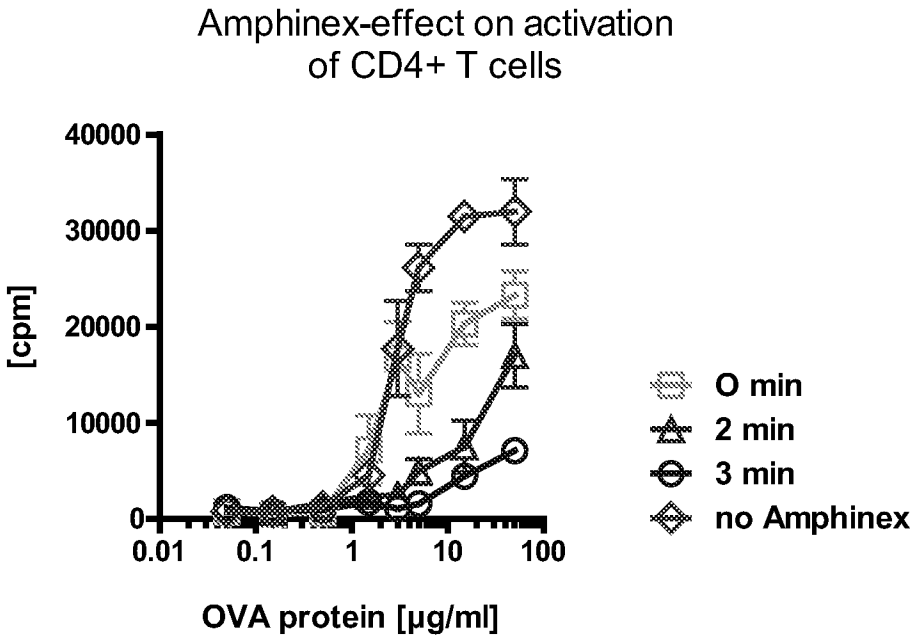
Amphinex-enhanced CD8+ T cell activation using primary mouse dendritic cells (BMDCs), Amphinex and antigen in an identical CD8+ T cell assay as described in Figure 2.

As with BMDMs and macrophage cell lines, we found an Amphinex-enhanced activation of specific CD8+ T cells. A 50-100-fold lower peptide concentration was required when BMDCs were treated with Amphinex + 3 min blue light. BMDCs seem to be more sensitive to Amphinex + light compared to macrophages, the Optimum effect was seen with 3 min light (macrophages: 5 min). Even “background” light (Amphinex + 0 min blue light) seems to have an effect on BMDCs. With 5 min light we saw no positive effect and at 8 min all BMDCs seemed to be dead (no T cell activation). A possible explanation for these findings might lie in the more fragile structure of dendritic cells compared to macrophages.

2. Amphinex does not enhances activation of antigen-specific CD4+ T cells

For testing of the Amphinex-effect on MHC class II antigen-presentation and CD4+ T cell activation, APCs were incubated with Amphinex and ovalbumin as described in the methods in an antigen-specific T cell setting with an ovalbumin-specific (OVA 323-339) CD4+ T cell clone.

Figure 4: Effect of Amphinex on antigen-specific CD4+ T cell activation



Primary mouse BMDMs and ovalbumin protein +/- Amphinex were used to stimulate ovalbumin-specific CD4+ T cells. APCs were incubated with 0.2 µg/ml Amphinex overnight. Amphinex was then washed away and APCs incubated with different concentrations of antigen for 4h before light-treatment. CD4+ T cells were added overnight and IL-2 produced from activated T cells was analyzed from the supernatant.

Primary BMDMs were used as APCs in these experiments as the macrophage cell lines failed to induce activation of CD4+ T cells with ovalbumin in this setting. Whole ovalbumin protein was used in these assays as for stimulation with OVA 323-339 peptide very high peptide concentrations were necessary (also no positive effect of Amphinex when peptide was used, data not shown). The figure shows the results from one out of two independent experiments with the same outcome.

Amphinex was found to have a negative effect on CD4+ T cell activation. We found a decrease in CD4+ T cell activation with Amphinex + light compared to stimulation with OVA protein alone. 0.2 µg/ml Amphinex + 3 min of blue-light completely abolished MHC class II antigen-presentation and CD4+ T cell activation. Amphinex without light did not influence CD4+ T cell activation.

It is clear from these results that MHC class II T cell presentation and CD4+ T cell activation do not benefit from Amphinex. In contrast: Amphinex + light decreases / completely inhibits CD4+ T cell activation.

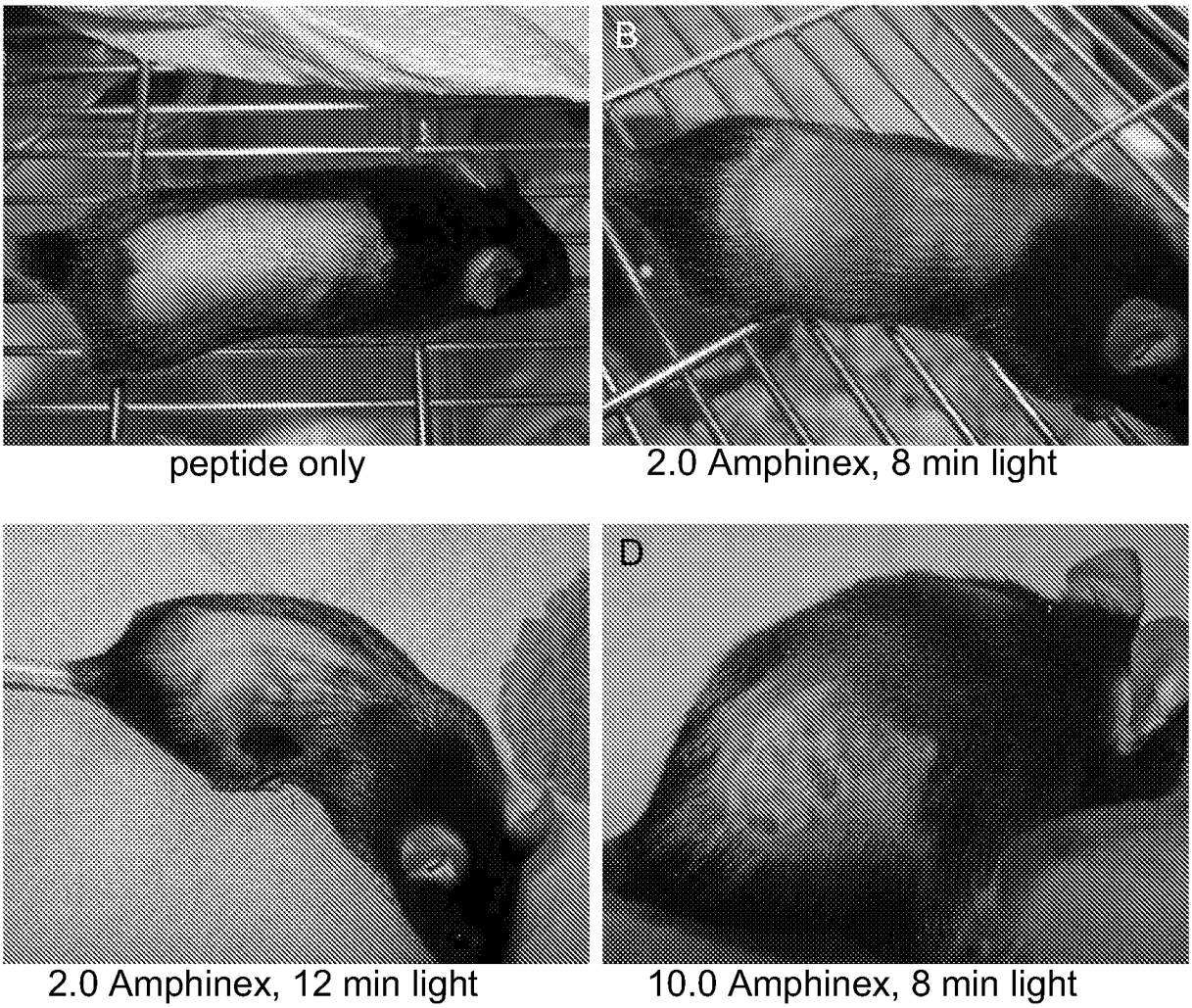
B. IN VIVO EXPERIMENTS

Amphinex enhances antigen-specific CD8+ T cell response *in vivo.*

The protocol chosen for the *in vivo*-studies was as follows:
- intra-dermal (i.d.) injection of mice with antigen (ovalbumin-peptide 100 µg in 100µl)
- co-injection of antigen and Amphinex
- no use of adjuvant
- light-treatment of the injection site approx. 20h post immunization
- a single injection of antigen +/- Amphinex
- detection of specific effector cells 6-10 days post immunization
- read-out: Multi-color flow-cytometry of spleen and/or blood cells. Staining for antigen-specific T cells using peptide-loaded MHC class I multimers (H-2Kb/SIINFEKL Pro 5 Pentamer PE, ProImmune, UK), binding exclusively the T cell receptor of CD8+ T cells specific for the peptide that was used for immunization; further phenotyping using fluorescent antibodies.

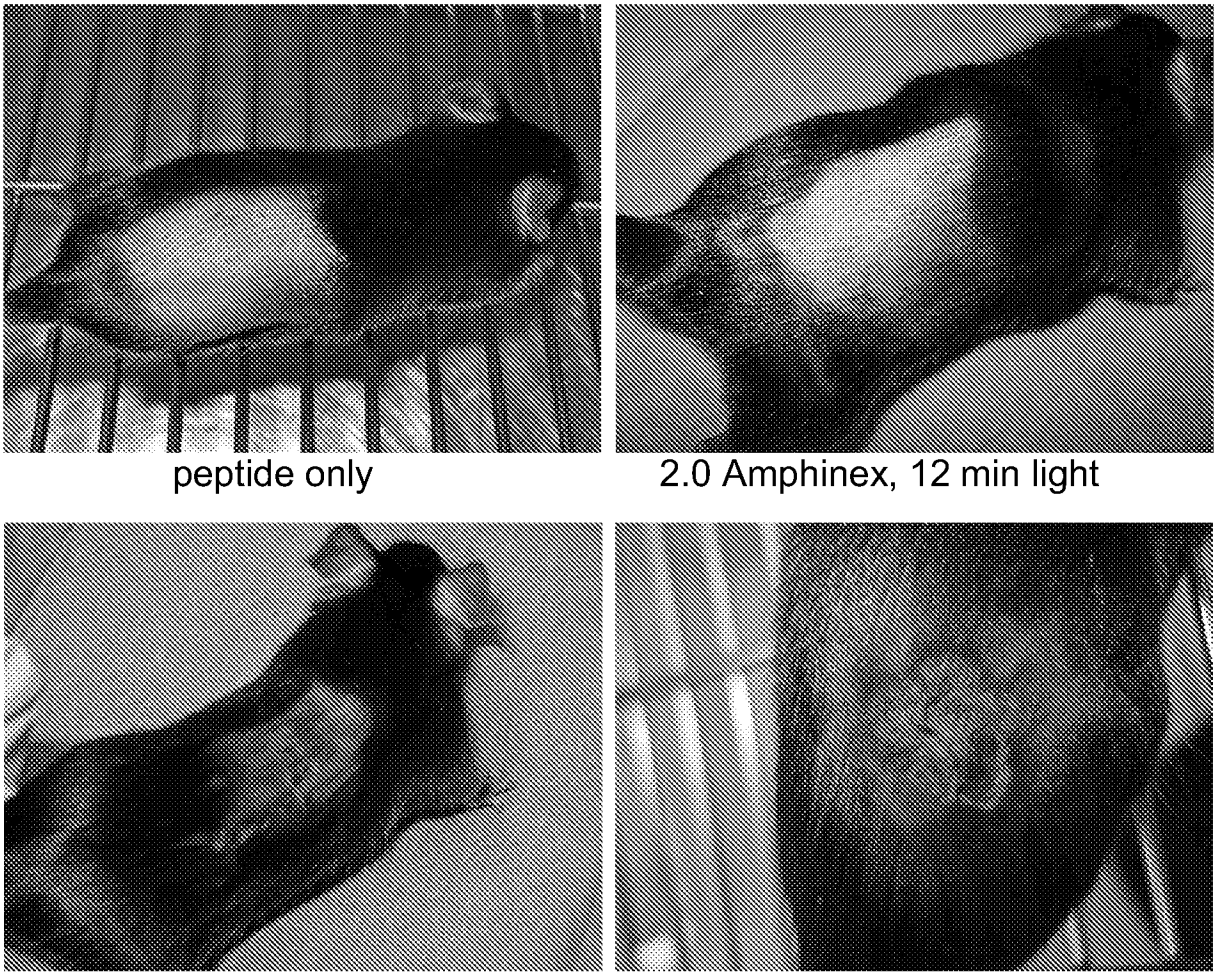
1. Tolerance of i.d. immunization with Amphinex and blue-light treatment

Figure 5: Immunized mice 24h after blue-light treatment.



Mice receiving peptide only (A) or Amphinex 2.0 µg/ml and 8 min blue-light (B) did not show significant side-effects / skin irritation 24h post immunization. Combinations of lower light and Amphinex-doses never resulted in skin irritations (not shown). Clear side-effects became visible when mice were longer exposed to light (C) or received a higher dose of Amphinex (D); signs of "sunburnt" skin. The animals were immunized as described in the methods section.

Figure 6: Immunized mice 72h after blue-light treatment.



Open wounds at high Amphinex-dose (10 µg/ml) with 8 min light (both pictures in the lower row). Wounds were healing from day 5 and were closed around one week post immunization.

The following conclusions were drawn:

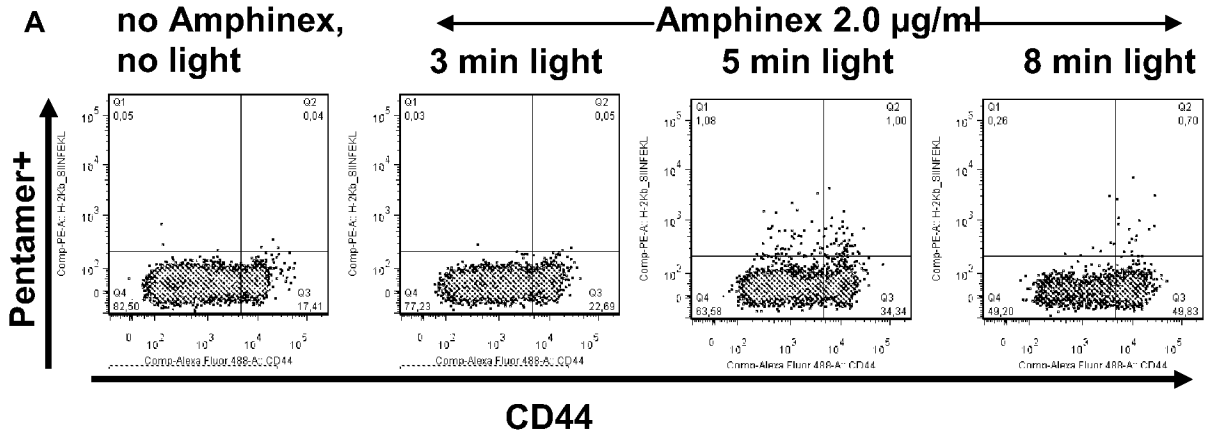
- Mice tolerate i.d. immunization with Amphinex + light well.
- No negative effects on skin seen with 2.0 Amph. + 8 min light.
- Few skin irritations were observed with higher light-doses (12 min, 2.0 Amph).
- Severe effects with open wounds 72h post immunization were seen with 5-fold increased Amphinex-concentration (10.0 Amph, 8 min light).

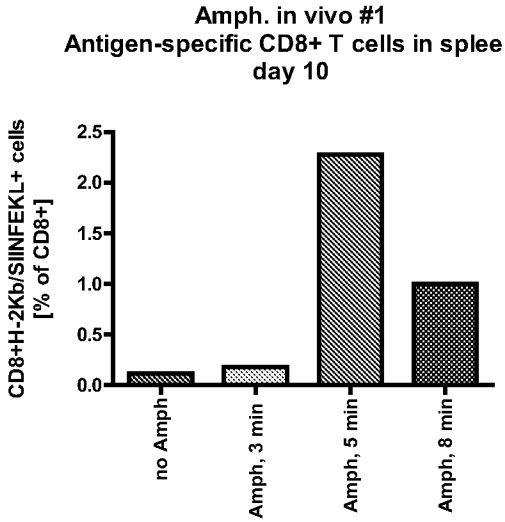
Thus, the chosen combination of Amphinex 2.0 µg/ml + 5 or 8 min blue light is tolerated well by the mice and seems to be just below the threshold of both light and Amphinex-dose where mice start suffering from side-effects. This combination is very promising.

2. Analysis of spleen cells 10 days post-immunisation with antigen and Amphinex followed by irradiation

The protocol used was as follows:
Day -1: 5 groups of mice (2 per group), anaesthesized and immunized i.d. with Amphinex and antigen as described in the methods.
Day 0: Blue-light: 20h after immunization: 0, 3, 5, 8 min (+ without Amphinex).
Day 1-5: mice were kept in the dark and inspected daily.
Day 10: Mice were killed and spleen cells analyzed.

Figure 7: Flow-cytometric analysis of splenocytes 10 days post-immunization.





A) Flow cytometry of isolated splenocytes stained with anti-CD8, anti-CD44 and MHC class I-Pentamer (H-2Kb/SIINFEKL Pentamer PE). Approximately 10^6 cells per sample were stained. Cells were gated for living splenocytes (FSC/SSC) and CD8+ T cells and analyzed for the activation marker CD44 and antigen-specificity to the OVA-peptide (MHC II-Pentamer-staining).
B) Bars show the percentages of OVA-specific T cells (CD8+Pentamer+ T cells) in mouse spleens 10 days after immunization with Amphinex +/- light.

We saw that mice perfectly tolerate at least 2.0 µg/ml Amphinex and 8 min blue-light. We found increased numbers of antigen-specific T cells with activated phenotype (CD8+CD44+Tetramer+) in spleens 10 days post-immunization from Amphinex-injected mice treated for 5 and 8 min with light.

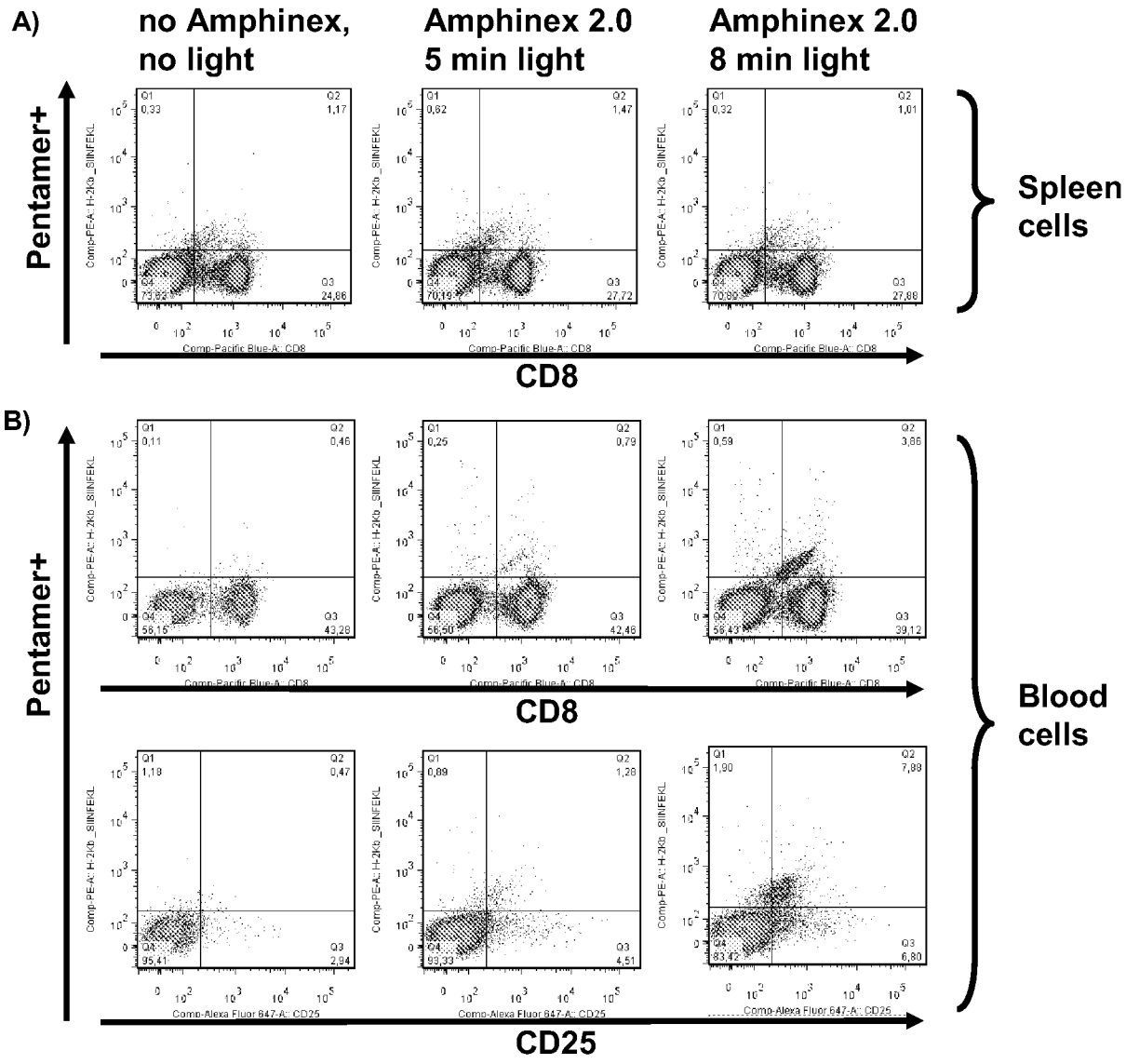
With Pentamer-staining we found a specific CD8+ effector T cell population in spleens 10 days post immunization. This population was only observed in the mice treated with Amphinex and 5 or 8 min light. The dose of Amphinex (2.0 µg/ml) was found to be effective and we therefore decided to use only a single i.d. immunization with 2.0 µg/ml Amphinex and OVA I peptide (antigen and Amphinex at the same time-point, 20h before treatment with different light-doses) as the protocol for the next experiment.

3. Analysis of blood and spleen cells 7 days post-immunisation with antigen and Amphinex followed by irradiation

The protocol used was as follows:
Day -1: mice, anaesthesized, shaved and i.d. immunized as described in the methods.
Day 0: Blue-light treatment: 20h after immunization: 3, 5, 8 min
Day 1-5: mice were kept in the dark and inspected daily.
Day 7: mice were killed and blood and spleen cells were isolated and analyzed by flow-cytometry.

The following materials were used:
- 20 mice
- 5 groups of 4 mice
- intradermal injection as described above (total 100 µl)
- antigen for all mice: 0.1 mg OVA I peptide
- +/- Amphinex: 2.0 µg/ml or positive control Resiquimod (R848))
- no Amphinex, Resiquimod (R848), Amph + 3 min, Amph. + 5 min, Amph + 8 min
- analysis: Detection of OVA-specific CD4+ T cells in blood and spleen 7 days post-immunisation

Figure 8: Flow-cytometric analysis of antigen-specific T cells in spleen and blood 7 days post-immunization

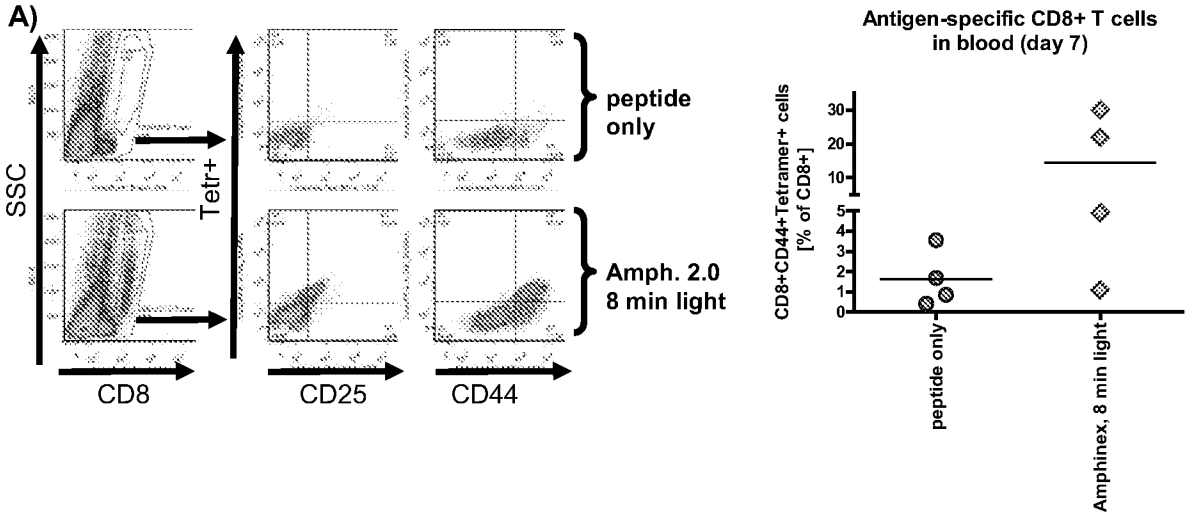


Staining procedure for blood and spleen cells was identical: lysis of red blood cells, cell count, staining of approximately 10^6 cells for CD8 (eFluor450), CD3 (FITC), CD25 (A647), and H-2Kb/SIINFEKL Pentamer (PE), CD19 (PE/Cy7). Cells were gated for living leucocytes (FSC/SSC), B cells were excluded (gate on CD19-negative population, B cells can cause non-specific Pentamer-staining) and gated for T cells (CD3+ cells). Left dot-plots: peptide only, middle: Amphinex + 5 min. light, right: Amphinex + 8 min light.
A) Spleen cells. One representative example is shown (first out of 4 mice per group). Antigen-specific CD8+ T cells are found in the upper right quadrant of the dot-plots.
B) Blood cells. Blood samples were pooled from all 4 mice per group due to the limited amount of Pentamer. Upper row: CD8 versus Pentamer as in A). Lower row: Cells were gated in addition for CD8+. Pentamer-staining versus expression of the activation-marker CD25 is shown (activated, specific CD8+ T cells are found in the upper right quadrant).

Re-analysis of blood cells for further parameters:
Antigen-specific CD8+ T cells are found in the blood but not in the spleen 7 days post-immunization. We were however not sure if this population is really antigen-specific or an artifact due to non-specific binding ("unusual" shape of the CD8+Pentamer+ population) and further tests were therefore conducted.

We repeated the flow-cytometry with individual staining for blood samples from the "peptide only" and "Amphinex/8 min light" group. We used antibody staining for the activation markers CD25 and CD44 to provide further evidence that we really detect antigen-specific CD8+ T cells in the blood of mice immunized with Amphinex.
Staining procedure of blood cells as described in Figure 8. CD3 antibody was replaced by staining for the activation marker CD44, CD19+ cells were excluded.

Figure 9: Amphinex-induced antigen-specific CD8+ T cell population in the blood of immunized mice shows an activated phenotype.



A) Staining examples of blood cells from peptide only (upper row) or Amphinex+ peptide (lower row) immunized mice. Left dot-plots: Gating on CD8+ T cells. A SSC-high/CD8 high-population is seen in the Amphinex-immunized mouse. Right dot-plots: CD8+-gated cells were analyzed for antigen-specificity (Pentamer, y-axis) and expression of the activation-markers CD25 and CD44 (x-axis). The Pentamer-positive, antigen-specific CD8+ population was found to be positive for both activation markers. This strongly argues in favor of detecting a really antigen-specific CD8+ population in mice immunized with Amphinex + light as adjuvant.
B) The results from the example-dotplots in A) are summarized for all analyzed samples (numbers of CD8+CD44+Pentamer+ cells from the upper-right quadrants in the dot-plots).

High numbers of activated, antigen-specific CD8+ T cells (>5% of CD8+ T cells in the blood) were found in the blood in 3 out of 4 mice immunized with Amphinex + 8 min light (but none of the mice injected with peptide alone).

From the results it is evident that immunization with OVApeptide and Amphinex + light can induce activation of antigen-specific CD8+ T cells.

7 days post infection: enhanced numbers of antigen-specific CD8+ T cells are found in the bloodstream but not in the spleen in significant numbers (compared to their presence at 10 days). This increase in CD8+ T cells in the bloodstream was dramatic. We found in 3 out of 4 mice more than 5% specific T cells (but none of the mice receiving peptide alone).

Amphinex 2.0 µg/ml + 8 min blue-light yielded the best results.

We re-stained blood cells with different markers to ensure that antigen-specific T cells were detected. The detected CD8+Pentamer-positive cell population was found to be highly positive for CD44 as well as CD25 but negative for CD19 thus we could clearly show that the cells have an activated phenotype (and exclude that we detect non-specific staining of B cells). In addition the specific CD8+ T cells represented a distinct population (FSC/SSC) with a larger and more granular phenotype. From these findings we conclude that we indeed detect an antigen-specific T cell population.

4. Analysis of blood and spleen cells 6 and 22 days post-immunisation with antigen and Amphinex followed by irradiation

We wanted to repeat the detection of enhanced antigen-specific CD8+ T cell numbers in the bloodstream around day 7 post immunization. We decided to analyze blood and spleen cells at 2 time-points post infection. As Amphinex 2.0 µg/ml + the highest light-dose (8 min) seems to be most effective and mice seem to perfectly tolerate this treatment, we wanted to test higher doses of Amphinex (10 µg/ml) and higher light-doses (12 min).

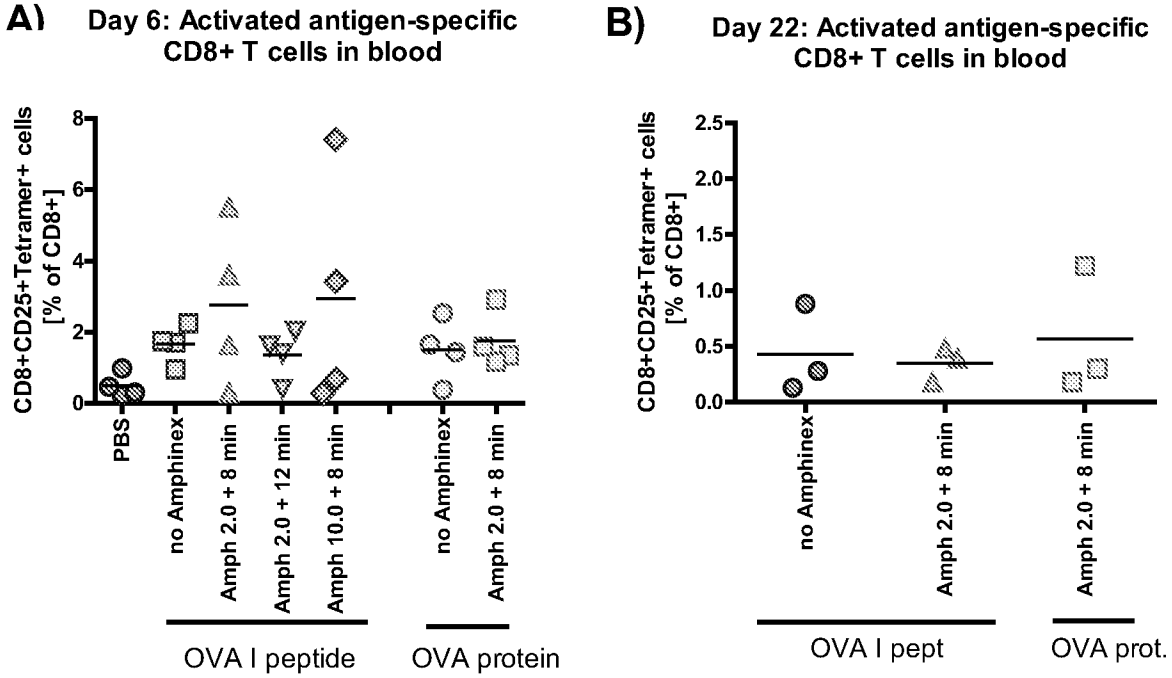
The procedure used:
- 40 mice
- 10 groups of 4 mice
- intradermal injection as described above (total 100 µl)
- OVA I peptide 100 µg and OVA protein 100 µg and i.d. were used
- 5 x higher dose Amphinex (10.0 µg/ml) was compared to Amphinex: 2.0 µg/ml.
- light-dose: 8 and 12 min
- re-injection of antigen (booster immunization) with 3 groups of mice

Day -1: I.d. immunization of the mice with antigen +/- Amphinex
Day 0: Blue-light: 20h after immunization: 8 and 12 min.
Day 1-5: mice were kept in the dark and inspected daily
Day 6: mice groups 1-7 were killed and blood and spleen cells were isolated and analyzed by flow-cytometry as in the other experiments.
Groups 8-10: Booster immunization day 8 and 14, analysis on day 22
Day 8: booster immunization mice group 8-10 (same procedure as on day -1 and 0).
Day 14: booster immunization mice

Day 22: mice groups 8-10 were killed and blood and spleen cells were isolated and analyzed by flow-cytometry as in the other experiments.

Mice developed open wounds with 10.0 µg/ml Amphinex (see methods, Figure 1). Wounds were healing after a few days (no open wounds seen on day 6).
Spleen and blood cells were analyzed 6 days post immunization:

Figure 10: Antigen-specific CD8+ T cell numbers in blood



Results from the flow-cytometric analysis of blood cells 6 and 22 days post-immunization is shown (percentages of CD8+CD25+Pentamer+ T cells, same experimental conditions as described in Figure 8).
A) Antigen-specific CD8+ T cells in blood of mice 6 days post immunization.
B) Antigen-specific CD8+ T cells in blood of mice 22 days post immunization (booster immunization on day 8 and 14).

As in the previous experiment we found enhanced numbers of specific T cells with Amphinex 2.0 and 8 min light (also with Amphinex 10.0 and 8 min light). The results from *in vivo* experiment Nr. 3 were thus reproducible.

No increased T cell numbers were seen when more light was used (12 min) and no effect was seen for OVA protein +/- Amphinex. No antigen-specific CD8+ T cells were detected 22 days post infection (neither with nor without Amphinex). No significant population of antigen-specific CD8+ T cells was found in spleen of mice on day 6 and day 22 (not shown).

In order to characterize further parameters that indicate an antigen-specific T cell response, we analyzed proliferation of CD8+ T cells in response to antigen on (day 22, CFSE-dilution). These results are not shown here, but summarized in brief:

Proliferation assay with spleen cells 22 days post immunization: Strong CD8+ proliferation in response to OVA-antigen was seen with Amphinex-immunized mice (peptide- as well as protein-immunized mice) but not with peptide only immunized mice.

CONCLUSIONS

Conclusions from the *in vitro* experiments:

An “adaptive immunostimulatory function” for Amphinex was found as Amphinex enhanced CD8+ T cell activation 10-100-fold compared to stimulation with peptide alone. The best effect was seen with 0.2 µg/ml Amphinex + 5 min.

Amphinex did not enhance MHC class II presentation or CD4+ T cell activation.

Conclusions from the *in vivo* experiments:

Mice perfectly tolerate intra-dermal immunization with 2 µg/ml Amphinex and 8 min blue-light treatment. This was also the most effective combination in these experiments.

Amphinex 2 min + 8 min light: no side-effects but positive effect on T cell activation.

There is evidence that Amphinex 2 min + 8 min light enhances CD8+ T cell activation *in vivo*.